

# The Autophagy-related Gene 14 (Atg14) Is Regulated by Forkhead Box O Transcription Factors and Circadian Rhythms and Plays a Critical Role in Hepatic Autophagy and Lipid Metabolism<sup>\*[5]</sup>

Received for publication, August 21, 2012, and in revised form, September 12, 2012. Published, JBC Papers in Press, September 19, 2012, DOI 10.1074/jbc.M112.412569

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**Background:** Atg14 is critical for the autophagy initiation.

**Results:** Our data show that the Atg14 gene can be regulated by FoxO and Clock transcription factors, and it has striking impacts on hepatic autophagy and triglycerides.

**Conclusion:** Atg14 is controlled by FoxOs and circadian rhythms, and it modulates hepatic lipid homeostasis.

**Significance:** These findings suggest that Atg14 is crucial for hepatic autophagy and lipid metabolism.

Autophagy plays a critical role in cell survival from prolonged starvation and recycling of aggregated proteins and damaged organelles. One of the essential genes involved in the autophagic initiation is autophagy-related 14 (Atg14), also called Barkor for Beclin 1-associated autophagy-related key regulator. Although its crucial role in the autophagic process has been reported, the gene regulation of Atg14 and its metabolic functions remain unclear. In this work we have identified that the Atg14 gene is regulated by forkhead box O (FoxO) transcription factors and circadian rhythms in the mouse liver. Luciferase reporter analyses and chromatin immunoprecipitation assays have revealed well conserved cis-elements for FoxOs and Clock/Bmal1 in the proximal promoter of the Atg14 gene. To examine the functions of hepatic Atg14, we have performed the gene knockdown and overexpression in the mouse livers. Remarkably, knockdown of Atg14 leads to elevated levels of triglycerides in the liver and serum as well. Conversely, overexpression of Atg14 improves hypertriglyceridemia in both high fat diet-treated wild-type mice and FoxO1/3/4 liver-specific knock-out mice. In summary, our data suggest that Atg14 is a new target gene of FoxOs and the core clock machinery, and this gene plays an important role in hepatic lipid metabolism.

Autophagy, an intracellular degradation process, has been shown to play a critical role in multiple biological functions, including cell survival, neuronal function, mitochondrial turnover, protein degradation, and energy metabolism (1). About 30 genes that were initially identified to be involved in autophagy in budding yeast *Saccharomyces cerevisiae* are collectively

named autophagy-related genes (Atg).<sup>2</sup> Many of these genes have homologs in higher organisms, and Atg14 (also known as Barkor for Beclin 1 (Becn1)-associated autophagy-related key regulator) is one of them (2, 3). Atg14 is part of a protein complex that is also composed of Beclin 1, vacuolar sorting protein 15 (Vps15, also called p150 or Pik3r4 (phosphatidylinositol 3 kinase, regulatory subunit, polypeptide 4)), and Vps34 (also named as Pik3c3, the catalytic subunit of the class III phosphatidylinositol 3-kinase), and this Atg14-containing complex plays an important role in the initiation process of autophagy (2–4). It has been characterized that Atg14 can recognize and bind to membrane curvatures through an interaction with phosphatidylinositol 3-phosphate in the bilayer membrane during the formation of preautophagosomes or omegasomes (5–7).

With better understanding of the fundamental process of autophagy, its pathophysiological functions have begun to be appreciated (8). For example, mice deficient in several key autophagy genes such as Atg5, Atg7, and Vps34 have elevated triglyceride levels in the liver (9–11), and overexpression of Atg7 can decrease hepatic triglycerides (11). This lipid breakdown process through autophagy is called lipophagy (10). Because Atg14 is involved in the autophagy-specific Vps34-containing complex and it is upstream of Atg5 and Atg7, we were curious about whether Atg14 has a role in lipid metabolism. Moreover, how the Atg14 is transcriptionally regulated is not yet clear. In this report we have identified a molecular mechanism for the Atg14 gene regulation and have demonstrated its critical role in hepatic lipid metabolism.

## EXPERIMENTAL PROCEDURES

**Animal Procedures**—C57BL/6J mice were purchased from The Jackson Laboratory (Bar Harbor, ME). FoxO1/3/4-LTKO mice were generated and genotyped as previously described (12). High fat diet (60% calories from fat) was purchased from

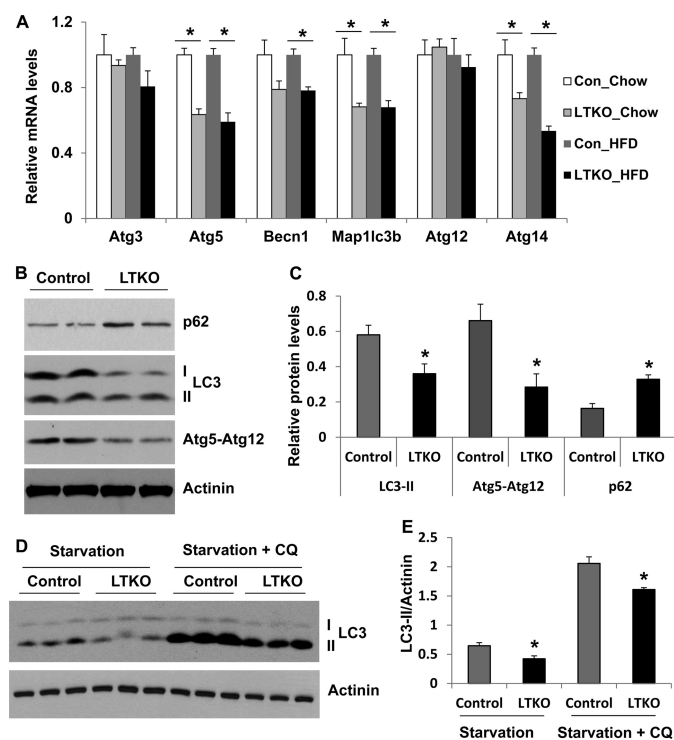
<sup>\*</sup> This work was supported, in whole or in part, by National Institutes of Health Grants R00DK077505 and R01DK091592 (NIDDK; to X. C. D.).

<sup>[5]</sup> This article contains supplemental Table S1.

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<sup>2</sup> The abbreviations used are: Atg, autophagy related; Vps34, vacuolar protein sorting 34; FoxO, forkhead box O; p62/Sqstm1, sequestosome 1; LTKO, liver-specific knock-out mice; IRE, insulin response element.

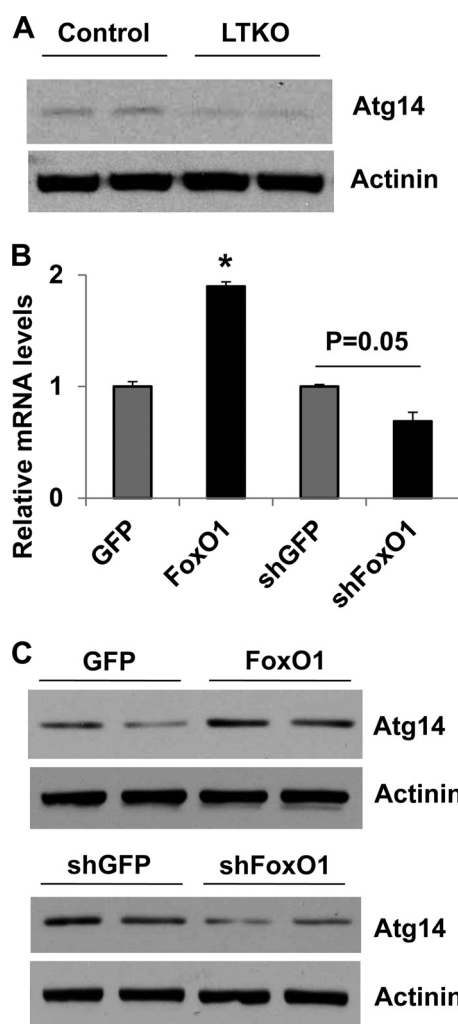
## The Atg14 Gene Regulation and Function



**FIGURE 1. Autophagy is impaired in FoxO1/3/4-deficient livers.** A, expression of several genes involved in autophagy was analyzed by real-time PCR in the liver of control (Con) and LTKO mice ( $n = 4-6$ ) fed either chow or a high fat diet (HFD) for 5 months after an overnight 16-h fasting. Data (mean  $\pm$  S.E.) are presented as relative values by normalizing to controls. B, autophagy indicators were examined in the livers of 2-month control and LTKO mice ( $n = 4-5$ ) that were fasted for 24 h by Western blot analysis. C, shown is a quantitative representation of the data in Panel B. D, primary hepatocytes were isolated from control and LTKO mice and cultured in regular DMEM medium for 24 h. Then cells were treated with Earle's balanced salt solution buffer in the presence or absence of 25  $\mu$ M chloroquine (CQ) for 1 h. Cell lysates were analyzed by Western blots. E, shown is a densitometric analysis of the relative LC3-II levels in Panel D. Data are presented as the mean  $\pm$  S.E. \*,  $p < 0.05$ .

Harlan Laboratories (Madison, WI). Circadian studies were performed as previously described (13). Serum and liver triglycerides were analyzed as previously described (12). Adenoviruses carrying Atg14, GFP, Atg14-shRNA, and GFP-shRNA were injected into mice at a dose of  $1-2 \times 10^9$  pfu via tail vein. All animal procedures were performed in accordance with the Guide for Care and Use of Laboratory Animals of the National Institutes of Health and were approved by the Institutional Animal Use and Care Committee of the Indiana University School of Medicine.

**Cell Culture**—Human HEK293A cell line was purchased from Invitrogen, and they were maintained in DMEM medium containing 100 units/ml penicillin and 100  $\mu$ g/ml streptomycin, 4.5 g/liter glucose, and 10% FBS. Mouse primary hepatocytes were isolated and cultured as previously described (12). The isolated primary hepatocytes were cultured in 4.5 g/liter glucose containing DMEM plus 10% FBS and antibiotics. For starvation conditions, cells were cultured in Earle's balanced salt solution (5.33 mM KCl, 26.19 mM NaHCO<sub>3</sub>, 117.24 mM NaCl, 1.01 mM NaH<sub>2</sub>PO<sub>4</sub>, 5.56 mM D-glucose) for 1 h. For GFP-LC3 dot counting, cells were cultured on coverslips, fixed with 4% paraformaldehyde for 15 min at 4°C, and then washed 3 times with PBS. Coverslips were mounted using the FluoroGel mounting medium (GeneTex) and viewed under fluorescent

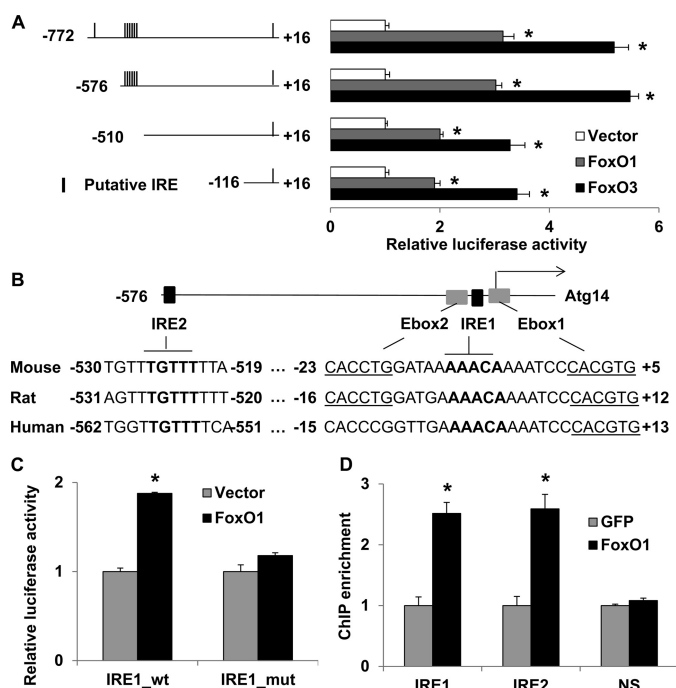


**FIGURE 2. FoxO1 modulates the Atg14 gene expression.** A, Atg14 protein was analyzed in the liver of control and LTKO mice by Western blots. B and C, Atg14 mRNA and protein analyses in mouse primary hepatocytes transduced with adenoviruses that carried FoxO1 and GFP overexpression or knockdown (shRNA) constructs. Primary hepatocytes were incubated in the DMEM media containing 1 g/liter glucose and 0.5% FBS for 12 h before gene expression analysis. Data are presented as the mean  $\pm$  S.E. \*,  $p < 0.05$ .

microscope (Zeiss) with a magnification of 200 $\times$ . GFP-LC3 dots counting was performed as previously described (14). At least 50 cells from more than 10 fields were counted for statistical analysis.

**Adenovirus Preparation**—Adenoviruses carrying Atg14, FoxO1, or GFP were generated using the pAdEasy system (Agilent). Adenoviruses carrying shRNA sequences against Atg14, FoxO1, or GFP were generated using the BLOCK-iT system (Invitrogen). Adenoviruses were amplified in HEK293A cells and purified by CsCl gradient centrifugation. The viruses were titrated using a QuickTiter adenovirus titer immunoassay kit (Cell Biolabs) according to the manufacturer's manual. Generally, we used 25–50 multiplicity of infection (m.o.i.) for overexpression and 100 m.o.i. for shRNA knockdown in mouse primary hepatocytes.

**Real-time RT-PCR**—Total RNA isolation was performed using TRIzol reagent (Invitrogen) and converted into cDNA using a cDNA synthesis kit (Applied Biosystems, Inc). Real-



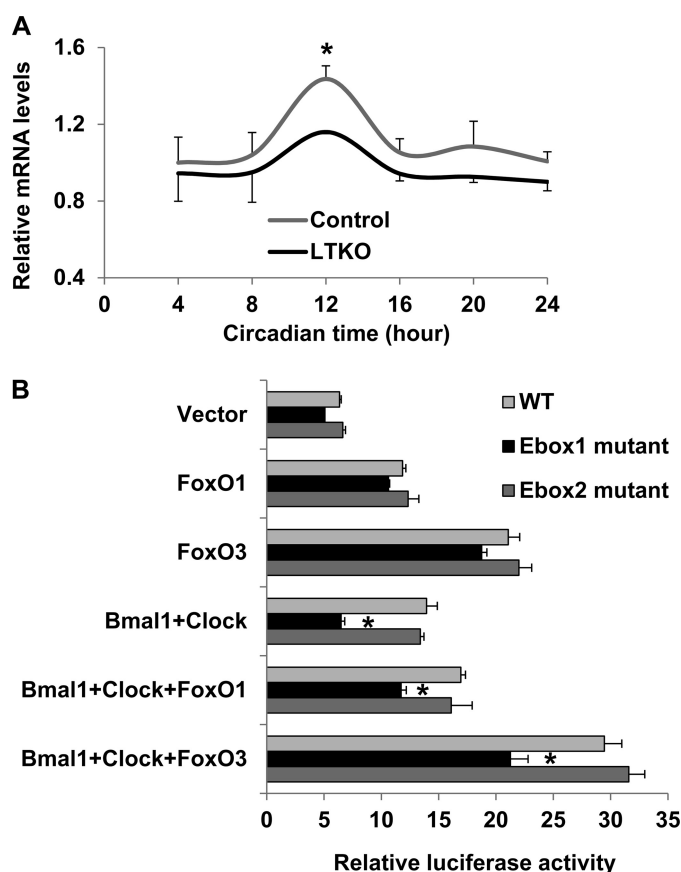
**FIGURE 3. The Atg14 gene is a direct target of FoxOs.** *A*, a schematic diagram of the proximal promoter of the mouse Atg14 gene is depicted on the left. The putative IREs are indicated by vertical lines. The nucleotide numbering is relative to the transcriptional start site. Data of luciferase reporter assays using serial deletion constructs of the mouse Atg14 gene promoter are presented on the right. *B*, shown is sequence alignment among mouse, rat, and human Atg14 gene promoters in the regions containing putative E-box and IRE elements. *C*, luciferase reporter assays were performed using the shortest promoter constructs that contain a wild-type (IRE1\_wt) or mutant IRE1 (IRE1\_mut) sequence. *D*, shown is ChIP analysis of the association between FoxO1 and the putative IRE1, IRE2, and an upstream nonspecific (NS) region by real-time PCR. Data are presented as mean  $\pm$  S.E. \*,  $p < 0.05$ .

time PCR analysis was performed using SYBR Green Master Mix (Promega) in an Eppendorf Realplex PCR system.

**Luciferase Reporter Assay**—Mouse Atg14 gene promoter was cloned by PCR using specific primers (see supplemental Table S1). Mutations in the Atg14 gene promoter were generated using the QuikChange II site-directed mutagenesis kit (Agilent). A firefly luciferase reporter system (pGL4.10luc2 and pGL4.74hRluc/TK) was purchased from Promega. DNA constructs were transfected into HEK293A cells, and luciferase activity was analyzed using a dual-luciferase assay system (Promega).

**Chromatin Immunoprecipitation (ChIP) Assay**—Mouse primary hepatocytes were cultured in the complete culture medium for 12 h before they were transduced with GFP or FoxO1 adenoviruses for 36 h. Then the cells were incubated with low glucose (1 g/liter) DMEM plus 0.5% FBS for 12 h before 1% formaldehyde was added for cross-linking. Chromatin DNA preparation and ChIP analysis were performed as previously described (12).

**Western Blot Analysis**—Tissue or cells were homogenized in the lysis buffer (50 mM Hepes, pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM MgCl<sub>2</sub>, 1 mM EGTA, 10 mM sodium pyrophosphate, 10 mM sodium fluoride, and freshly added 100  $\mu$ M sodium vanadate, 1 mM PMSF, 10  $\mu$ g/ml aprotinin, and 10  $\mu$ g/ml leupeptin). Protein extracts were resolved on an SDS-PAGE gel and transferred to nitrocellulose mem-



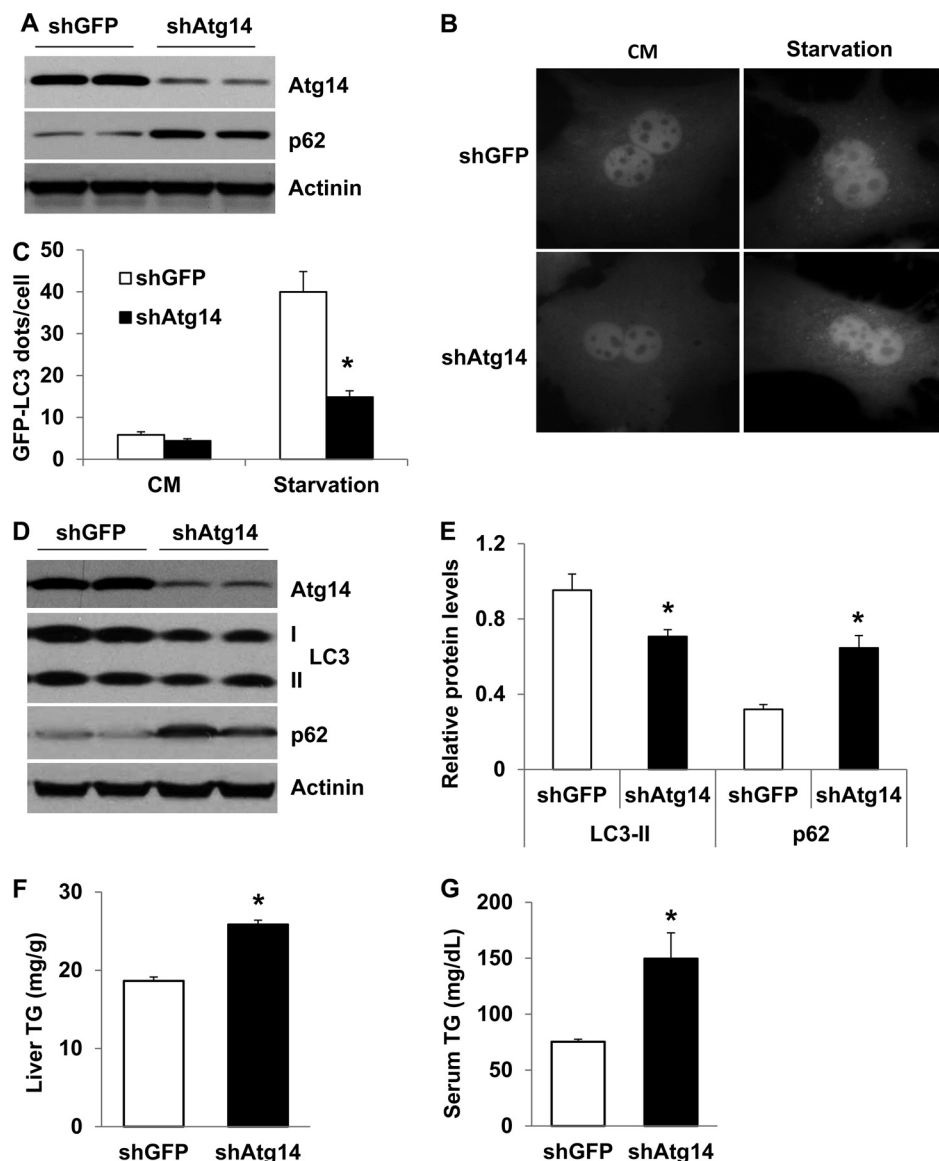
**FIGURE 4. The Atg14 gene is also regulated by the circadian clock.** *A*, shown are relative expression levels of Atg14 mRNA in the livers of 2-month control and LTKO mice under the condition of 24-h constant darkness ( $n = 3-4$  mice per genotype per time point). *B*, luciferase reporter assays using either wild-type or E-box mutated constructs of the mouse Atg14 gene promoter are shown. The reporter constructs were transfected into HEK293A cells along with control vector, FoxO1, FoxO3, or Bmal1 and Clock expression plasmids. Data are presented as the mean  $\pm$  S.E. \*,  $p < 0.05$ .

brane (Santa Cruz Biotechnology). Proteins were probed using the following antibodies: LC3B, p62 (Cell Signaling Technology), Atg14 (Sigma), actinin (Santa Cruz Biotechnology). Detection of proteins was carried out by incubations with HRP-conjugated secondary antibodies followed by ECL detection reagents (Pierce).

**Statistical Analysis**—All data are presented as the mean  $\pm$  S.E. Analysis was performed using 2-tailed unpaired Student's  $t$  test, and  $p < 0.05$  was considered as significant.

## RESULTS

**FoxO1/3/4 Deficiency Impairs Autophagy in the Liver**—Recently, we reported that FoxO1/3/4 liver-specific knock-out mice (LTKO) develop hepatic steatosis and hypertriglyceridemia (12). To examine whether lipophagy plays a role in this pathological phenotype, we analyzed autophagic events in the livers from control and LTKO mice that were fed chow or a high fat diet. The real-time PCR data showed that mRNA levels of Atg5, Becn1, Map1lc3b (LC-3B; microtubule-associated protein 1 light chain 3 $\beta$ ), and Atg14 genes, which are known to play critical roles in autophagy, were markedly lower in the LTKO mice relative to the controls (Fig. 1A). Next, we performed additional analysis of hepatic autophagy using three commonly



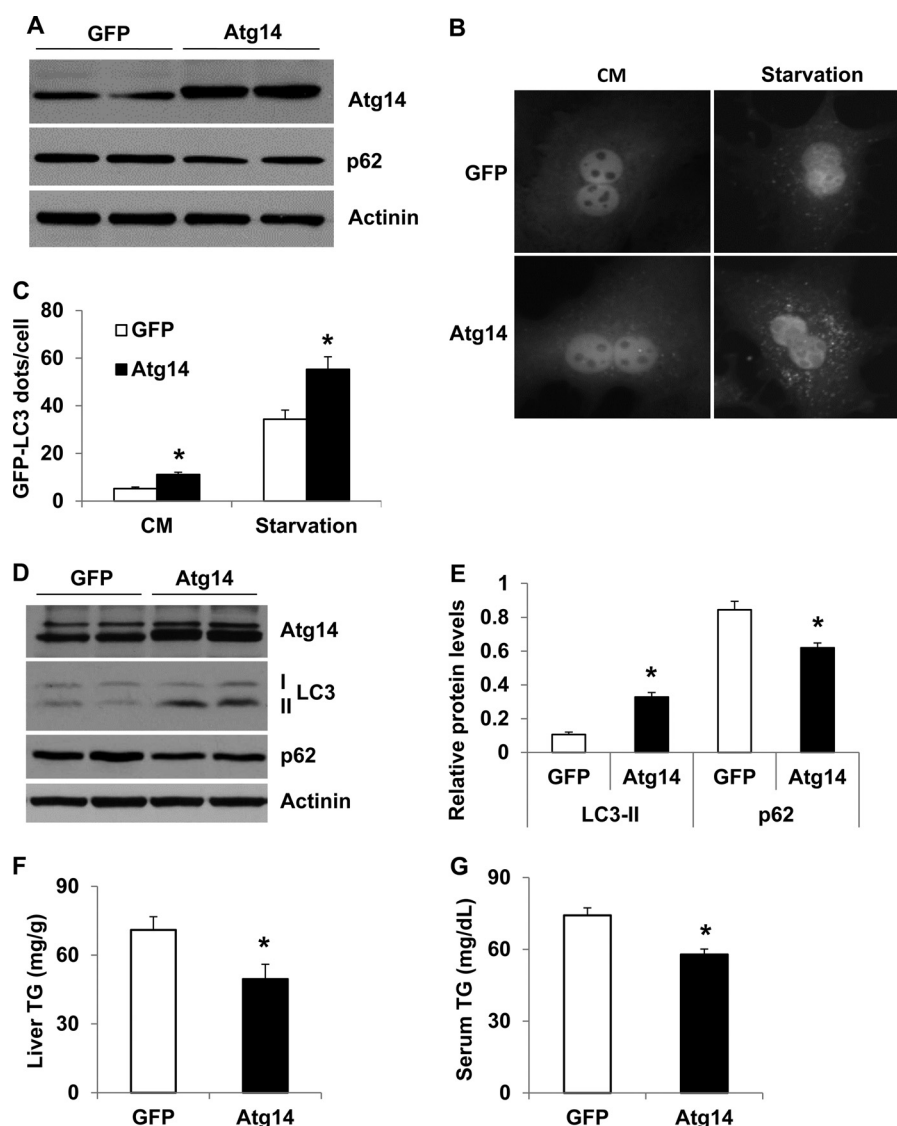
**FIGURE 5. The Atg14 gene knockdown attenuates hepatic autophagy and elevates triglyceride (TG) concentrations.** *A*, Western blot analysis of Atg14 knockdown is shown. Primary hepatocytes were transduced with Atg14 shRNA or control shGFP adenoviruses for 72 h and then cultured in the starvation medium for 1 h. Cell lysates were analyzed by Western blots. *B*, shown is fluorescent microscopy analysis of the Atg14 knockdown effect on autophagy. Primary hepatocytes were transduced with adenoviruses for shGFP or shAtg14 plus GFP-LC3 reporter. The cells were cultured in either complete medium (CM) or EBSS buffer for 1 h (Starvation). Then they were fixed with 4% paraformaldehyde for 15 min, and then GFP-LC3 signals were analyzed using a fluorescent microscope. *C*, quantification of GFP-LC3-positive dots per cell is shown. The data are the mean  $\pm$  S.E. from  $>50$  cells. *D*, immunoblot analysis of autophagy markers in the liver of 3-month wild-type mice injected with shAtg14 or shGFP adenoviruses is shown. *E*, quantification of the relative levels of LC3-II and p62 in *Panel D* is shown. *F* and *G*, liver and serum triglyceride levels were measured in the shGFP or shAtg14 adenovirus-infected mice ( $n = 5$ ). Data are presented as the mean  $\pm$  S.E. \*,  $p < 0.05$ .

used markers, p62/Sqstm1 (sequestosome 1), LC-3, and conjugated Atg5-Atg12 (14). The p62 protein is involved in chaperone-mediated protein degradation. During autophagy, the amount of p62 is inversely correlated with cellular autophagic activity (15). The Western blot data showed that the LTKO liver had an elevated level of p62 (Fig. 1, *B* and *C*), suggesting decreased autophagic activity in the LTKO liver. Another autophagy marker is LC3 that has two forms: LC3-I and LC3-II. When autophagy is activated, cytosolic LC3-I is converted to membrane-bound lipidated LC3-II (16). However, the amount of LC3-II in the LTKO livers significantly decreased (Fig. 1, *B* and *C*). The third indicator of autophagy is that Atg12 (a ubiquitin-like protein) is covalently attached to Atg5 during the

conjugation process (16). In the LTKO liver, the Atg5-Atg12 conjugation was decreased (Fig. 1, *B* and *C*). To more accurately monitor autophagic flux, we starved mouse primary hepatocytes in the absence or presence of chloroquine, which modifies lysosomal pH and, therefore, inhibits autophagy flux through lysosomes. Again, we noticed a significant lower autophagic activity in the LTKO hepatocytes (Fig. 1, *D* and *E*). Altogether, these data suggest autophagy is impaired when FoxOs are deficient.

**The Atg14 Gene Expression Is Regulated by FoxOs and Circadian Rhythms**—Because Atg14 is a critical player in the initiation of autophagy, we further investigated how it is regulated by FoxOs. First, we confirmed that the Atg14 gene was also down-



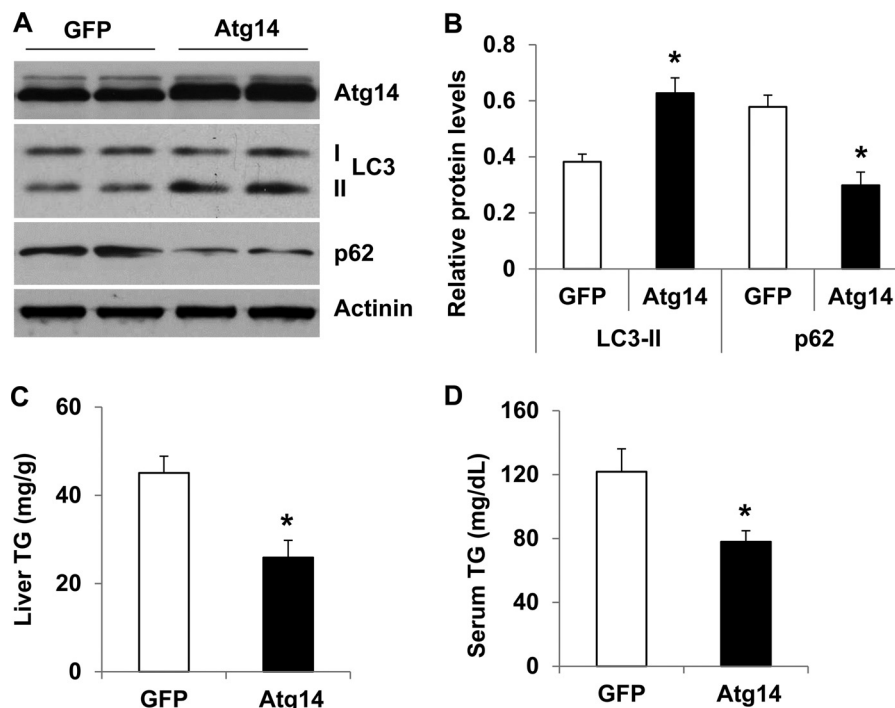


**FIGURE 6. Overexpression of Atg14 stimulates autophagy and lowers triglyceride (TG) concentrations.** *A*, Atg14 overexpression was assessed by Western blot analysis in mouse primary hepatocytes that were transduced with GFP or Atg14 adenoviruses. *B*, fluorescent microscopy analysis of the Atg14 overexpression effect on autophagy in mouse primary hepatocytes transduced with GFP or Atg14 plus GFP-LC3 adenoviruses is shown. *C*, shown is quantification of GFP-LC3-positive dots in >50 hepatocytes under the same conditions as in *panel B*. *CM*, complete medium. *D*, shown is a Western blot analysis of the impact of the Atg14 overexpression on autophagy in livers of wild-type mice treated with a high fat diet for 3 months followed by adenoviral overexpression of Atg14 ( $n = 5$ ) or GFP ( $n = 5$ ). *E*, shown is quantification of the relative levels of LC3-II and p62 in *Panel D*. *F* and *G*, liver and serum triglyceride levels were measured in the above GFP or Atg14-overexpressing mice ( $n = 5$ ). Data are presented as the mean  $\pm$  S.E. \*,  $p < 0.05$ .

regulated at protein levels in LTKO mice fed chow diet (Fig. 2A). Second, overexpression or knockdown of FoxO1 significantly altered expression of the endogenous Atg14 gene (Fig. 2, *B* and *C*). To assess whether the gene regulation is through an interaction with the promoter sequence, we made four serial deletion constructs that contain a different number of putative insulin response elements (IREs) in the mouse Atg14 gene promoter. By comparing luciferase activities between different promoter constructs, we identified one IRE cluster (between  $-576$  and  $-510$  bp) and a single IRE ( $-10$  to  $-6$  bp) that were largely responsible for the FoxO1- and FoxO3-regulated Atg14 promoter activities (Fig. 3A). Additionally, *in silico* analyses revealed two conserved IREs (IRE1 and IRE2) in the proximal promoters of human, mouse, and rat Atg14 genes (Fig. 3B). To validate whether the conserved IRE1 is involved in the Atg14 gene regulation, we made a mutation construct that lacked the

critical "CA" in the IRE1 DNA sequence (AAACA) and performed similar luciferase reporter assays. Indeed, the results revealed that the IRE1 was required for the FoxO1 action (Fig. 3C). Because there are six tandem putative IREs surrounding the conserved IRE2 in the mouse Atg14 gene promoter, we have not generated mutations to confirm that IRE2 is a functional FoxO binding site; however, the serial deletion mutants indicated that at least one of the IREs between  $-576$  and  $-510$  bp might be involved in the Atg14 gene regulation by FoxOs (Fig. 3A). Chromatin immunoprecipitation PCR also confirmed that FoxO1 was associated with the chromatin in the IRE1 and IRE2 regions (Fig. 3D). Altogether, these findings suggest that FoxOs positively regulate the Atg14 gene expression.

Recently it has been reported that some autophagy genes are controlled by circadian rhythms (17). We analyzed expression of the Atg14 gene in liver samples from control and LTKO mice



**FIGURE 7. Acute overexpression of Atg14 improves autophagy and lipid profiles in the LTKO mice.** *A*, shown is an immunoblot analysis of Atg14, LC3-II, and p62 protein levels in liver lysates from LTKO mice injected with Atg14 or GFP adenoviruses ( $n = 5$ ). *B*, shown is quantitative analysis of the LC3-II and p62 levels in *Panel A*. *C* and *D*, liver and serum triglyceride (TG) levels were measured in LTKO mice ( $n = 5$ ) infected with Atg14 or GFP adenoviruses. Data are presented as the mean  $\pm$  S.E. \*,  $p < 0.05$ .

under constant darkness, an experimental condition to assess the intrinsic rhythms. The results showed that whereas the rhythmicity was still maintained in the LTKO livers, the amplitude of the Atg14 gene expression was dampened, especially at the peak time (CT12) (Fig. 4A). During the Atg14 promoter sequence analysis, we also noticed two putative E-box elements that the core clock machinery Clock/Bmal1 heterodimer may bind to (Fig. 3B). To identify which E-box could mediate the Clock/Bmal1 transcriptional activation, we individually mutated two E-box elements and tested them in luciferase reporter assays. The results revealed that the proximal E-box 1 not 2 was responsible for the Clock/Bmal1 effect (Fig. 4B).

**Atg14 Modulates Hepatic Lipid Metabolism**—Although Atg14 has been shown to regulate autophagy in other cell types (2–6), not much is known about its role in the liver. We first examined the effects of the Atg14 gene knockdown in mouse primary hepatocytes. As expected, Atg14 deficiency impaired p62 turnover (Fig. 5A). To directly monitor the effect of Atg14 knockdown in cells, we introduced GFP-LC3 reporter along with shGFP or shAtg14 in mouse primary hepatocytes. The results showed that knockdown of the Atg14 gene dramatically reduced the number of LC3-associated puncta (Fig. 5, B and C). Next, we also knocked down the Atg14 gene in mouse livers using an adenovirus-mediated shRNA approach. Consistently, hepatic autophagy activity was also decreased in the Atg14 knockdown livers compared with the controls, indicated by lower LC3-II levels and higher p62 amounts (Fig. 5, D and E). Significantly, hepatic and serum triglyceride levels were increased by 40 and 50% in the Atg14-deficient livers relative to the controls, respectively (Fig. 5, F and G), suggesting that Atg14 has a critical role in lipid metabolism.

To further demonstrate that Atg14 indeed plays an important role in hepatic autophagy and lipid metabolism, we also overexpressed Atg14 in mouse primary hepatocytes and livers. As expected, overexpression of the Atg14 gene in the primary hepatocytes enhanced autophagic activities, indicated by a decrease in p62 and an increase in the number of GFP-LC3 positive dots (Fig. 6, A–C). Moreover, we injected Atg14-expressing adenoviruses into mice that had been fed a high fat diet for 3 months. Nine days later animals were sacrificed for blood and tissue collection. The Western blot data indicated that autophagy activity was increased in the Atg14-overexpressing (Ad-Atg14) livers compared with the control GFP group (Fig. 6, D and E). Hepatic and serum triglycerides were decreased in Ad-Atg14 mice by 30 and 20% relative to the Ad-GFP mice, respectively (Fig. 6, F and G).

Because the Atg14 gene expression is decreased in the liver of LTKO mice, we were also curious about whether overexpression of Atg14 could improve the autophagy defects and dyslipidemia in the LTKO mice. Indeed, the Atg14 overexpression significantly increased autophagic activity in the LTKO livers, which is indicated by increased LC3-II and decreased p62 protein (Fig. 7, A and B). Also importantly, hepatic and circulated triglycerides were reduced to nearly normal levels (Fig. 7, C and D). These data suggest that Atg14 plays a critical role in the FoxO-regulated autophagy and lipid metabolism.

## DISCUSSION

Depending upon stress conditions, autophagy is regulated in an acute or chronic manner. In the short term autophagy can be acutely modulated by the AMPK, Vps34, and mTOR pathways (1, 8), whereas for the long term adaptation, transcription of

autophagy-related genes is also involved. For example, in response to nutrient deprivation, numerous genes including Atg5, Bnip3 (BCL2/adenovirus E1B interacting protein 3), Gabarapl1 ( $\gamma$ -aminobutyric acid A receptor-associated protein-like 1), LC3, and Ulk1 (unc-51 like kinase 1) are induced at the transcriptional level (17–24). Several of them have been shown to be regulated by FoxO1 or FoxO3 in skeletal muscle, cardiac muscle, neurons, kidney, and the liver, respectively (20–23, 25–38). In cancer cells, FoxOs can modulate autophagy in either transcription-dependent or transcription-independent fashion (39, 40). In this report, we have identified Atg14 as a novel target of FoxOs that can activate autophagy in the liver. Atg14 is an evolutionarily conserved protein that exists in a variety of organisms from yeast to mammals. The Atg14 protein has two critical domain structures: the coiled-coil domain (CCD) in the N-terminal region and the Barkor autophagosome targeting sequence (BATS) domain in the C terminus (2, 4, 5). The coiled-coil domain is required for the interaction with Beclin 1, and the BATS domain is essential for Atg14 targeting to an autophagosome. Additionally, the Beclin 1-Atg14-Vps34 complex also stabilizes the Atg14 protein (2). The critical role of Atg14 in autophagy has been firmly established in cell-based systems or lower organisms (2, 4–7); however, its gene regulation and physiological functions in mammals are unclear. In this work we have attempted to address these questions.

Our initial study reveals that Atg14 is significantly down-regulated in the LTKO livers. Further *in vitro* and *in vivo* analyses confirm that FoxO1 and FoxO3 regulate the Atg14 gene through an interaction with the proximal IRE cis-elements. Interestingly, like Nampt (nicotinamide phosphoribosyltransferase), another FoxO target (12), Atg14 gene expression also exhibits circadian rhythm, which is also controlled by the core clock components, Clock/Bmal1. Recently, it has also been shown that some other autophagy genes such as Bnip3 and Gabarapl1 are regulated by circadian rhythms via C/EBP $\beta$  (CCAAT/enhancer binding protein  $\beta$  (C/EBP $\beta$ )) (17). Whether Atg14 is also under C/EBP $\beta$  control needs further investigation. Nevertheless, an integral regulation of the Atg14 gene by FoxOs and Clock/Bmal1 reveals an orchestration of feeding and circadian rhythms because FoxOs are tightly controlled by fasting/feeding cycles, and the Clock/Bmal1 complex is the core circadian machinery. The implication of the Atg14 gene regulation in hepatic physiology is also significant because autophagy plays such a fundamental role in cellular fitness and survival. For example, aged or damaged mitochondria require autophagy, also called mitophagy, for recycling (41).

In addition to general functions in cell survival, organelle turnover, and protein recycling, autophagy has also been shown to play a role in lipid breakdown (10, 42–45); however, the underlying molecular mechanism remains elusive (46–48). That Atg14 plays a critical role in autophagy and lipid metabolism as well suggests that normal autophagic functions may be required for lipid homeostasis. It is possible that the Atg14-containing protein complex might be involved in the early stage of autophagic engulfment of lipid droplets. Once autophagosomes are fused with lysosomes, the enclosed lipid droplets can be degraded for further metabolism. The critical question is whether Atg14 can be

detected near lipid droplets during the initiation of lipophagy process. However, we cannot rule out that Atg14 might have autophagy-independent functions in lipid metabolism as another autophagy-related gene, Atg2, can bind to lipid droplets and appears to impact their size and distribution in an autophagy-independent fashion (42). Thus, more research is needed to illustrate the mechanism for the Atg14-regulated lipid metabolism.

In summary, our data suggest that FoxOs and the circadian clock can regulate Atg14 gene expression, thereby promoting autophagy. Moreover, Atg14 also plays an important role in hepatic lipid homeostasis. These findings further reinforce the connection between insulin-FoxO pathway and autophagy and lipid metabolism processes.

**Acknowledgments**—We thank Min Li, Hao Zhang, and Lin Xu for technical assistance. We also thank Dr. Xiao-Ming Yin for helpful discussions.

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